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Antiviral Research 37 (1998) 131–140

Antiviral Research

Baicalin, an inhibitor of HIV-1 production in vitro

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Received 25 September 1996; accepted 8 December 1997

Abstract

The flavonoid baicalin markedly inhibits replication of human immunodeficiency virus type 1 (HIV-1) in a concentration-dependent manner in normal peripheral blood mononuclear cells (PBMC) stimulated with phytohemagglutinin (PHA) in vitro. The effect was more pronounced when the cells were pretreated with baicalin. Furthermore, baicalin inhibits HIV-1 replication in PHA-stimulated PBMC from asymptomatic HIV-1-seropositive carriers. The 50% inhibitory concentration for HIV-1 replication was approximately 0.5 μg/ml. At the concentration of 2 μg/ml of baicalin, copy numbers of HIV-1 proviral DNA were approximately 50 times less than in untreated controls. In a cell-free infection system, baicalin inhibited the activity of HIV-1 reverse transcriptase (RT), but not the activity of human DNA polymerases α and γ (DNA polymerase β was slightly inhibited), suggesting that the anti-HIV-1 effect of baicalin may at least partly be due to inhibition of HIV-1 RT.

Keywords: Baicalin; PBMC; Reverse transcriptase inhibition; HIV-1

1. Introduction

3′-Azido-2′,3′-dideoxythymidine (AZT), 2′,3′-dideoxyinosine (ddI), 2′,3′-dideoxycytidine (ddC), and 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T), are among the drugs that are currently used for the treatment of individuals infected with human immunodeficiency virus type 1 (HIV-1) (Mitsuya et al., 1985; Fischl et al., 1987; Browne et al., 1993; Yarchoan et al., 1986, 1988). These pyrimidine and purine nucleoside analogues are potent inhibitors of HIV replication in human T-cells and monocyte/macrophages. However, the use of

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0166-3542/98/$19.00
PII S0166-3542(97)00069-7

these compounds is associated with adverse side effects in patients (Richman et al., 1987) and the emergence of resistant variants of HIV-1 (Larder et al., 1989). Various attempts have been made to develop new antiretroviral agents (Merluzzi et al., 1990; Pauwels et al., 1990), as reviewed by De Clercq (1995a,b).

Several plant flavonoids such as baicalein, quercetin, quercetagetin and myricetin have been shown to inhibit HIV-1 reverse transcriptase (RT) activity (Ono and Nakane, 1990). Recently, baicalin (5,6,7-trihydroxyflavone-7-*O*-β-D-glucopyranosideuronic acid), has been reported to inhibit the replication of HTLV-I in productively infected T- and B-cell lines (Baylor et al., 1992). Here we report the inhibitory effect of baicalin on the production of HIV-1 in peripheral blood mononuclear cells (PBMC) from healthy donors (HIV-seronegative persons) and HIV-1-infected individuals.

2. Materials and methods

2.1. Compounds

Baicalin (TJN-151: Mw 464.38, Fig. 1) was extracted and purified from *Scutellariae radix* (Havsteen, 1983) by Tsumura & Co. with a purity of not less than 98%. AZT (Sigma, St. Louis, MO) was used as reference of anti-HIV agent.

2.2. Cell culture and virus source

PBMC were isolated by Ficoll-Hypaque centrifugation from healthy donors (Böyum, 1968). PBMC were suspended in RPMI-1640 medium containing 10% heat-incubated fetal calf serum (FCS), penicillin G (50 U/ml), streptomycin (50 μg/ml), L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), and phytohemagglutinin (PHA) (10 μg/ml) (Sigma) for 24 h. One strain of HIV-1 (HIV_{MN}) was used in the anti-HIV-1 assays. HIV_{MN} selected as virus clone was kindly supplied by the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD.

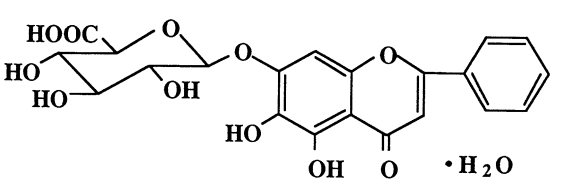

Fig. 1. Structural formula of TJN-151 (monohydrate of baicalin): 5,6,7-trihydroxyflavone-7-*O*-β-D-glucopyranosideuronic acid monohydrate.

2.3. Evaluation of anti-HIV-1 activity using fresh PBMC

In the presence of various concentrations of baicalin, 3×10^5 cells of PHA-activated normal PBMC were preincubated for 1 h and then exposed to 100 cell culture 50% infective dose ($CCID_{50}$) of HIV_{MN}. After 1-h virus adsorption, the cells were washed three times to remove non-adsorbed virus and suspended in culture medium supplemented with 40 U/ml of human recombinant IL-2 (Shionogi, Osaka, Japan) at 0.3×10^6 cells/ml. The cells were resuspended in 200 μl of fresh medium, and incubated in 96-well culture plates for 7 days. At 7 days postinfection, HIV-1 p24 antigen in the culture supernatant was determined by an enzyme-linked immunosorbent assay (ELISA; Abbott, North Chicago, IL) and the virus-inhibitory effect of baicalin was estimated (Barré-Sinoussi et al., 1983; Honda et al., 1989). The number of viable cells was determined by the Trypan blue dye exclusion test.

2.4. Evaluation of anti-HIV-1 activity in PBMC from HIV-1-infected individuals

HIV-1-seropositive blood specimens were identified through HIV-1 antibody ELISA testing (Abbott, North Chicago, IL), with confirmation by Western blot analysis (Bio-Rad, Richmond, CA). Then PBMC from 1100 specimens were tested for HIV-1 production. PBMC from five donors were selected for the assay, because they produced significantly higher amounts of HIV-1 p24 antigen (>1000 pg/ml) after PHA-stimulation for 7 days than PBMC from the other donors. The selected donors were asymptomatic

HIV-1 carriers with CD4 cell counts of less than 400/μl. Cultures containing 4×10^5 cells/ml of the PBMC were incubated for 7 days in the presence or absence of baicalin at various concentrations in 24-well culture plates, the supernatants were collected, and stored at -80°C until use. Production of HIV-1 was determined by HIV-1 p24 antigen ELISA. The number of cells was counted in a Multisizer (Coulter, Hialeah, FL) and the viability of the cells was determined by the Trypan blue dye exclusion method.

2.5. Polymerase chain reaction

HIV-1 DNA was detected by polymerase chain reaction (PCR). The reaction mixture contained 10 mM Tris–HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% (w/v) gelatin; 200 μM each of dATP, dCTP, dGTP and dTTP; 1 μM primers; 1 μg of template DNA; and 2.5 U of Taq polymerase in a total volume of 50 μl. PCR was performed in a Perkin–Elmer Cetus DNA Thermal Cycler by the following program: template denaturation, 1 min at 94°C; primer annealing, 2 min at 55°C; DNA synthesis by the Taq polymerase, 2 min at 72°C; 40 cycles. SK38/SK39 were selected as primer sequences and SK19 was prepared for detection of HIV-gag DNA. PCR products were subjected to agarose gel electrophoresis, transferred to nylon membranes, and hybridized with the radiolabeled HIV DNA probes. 8E.5 cells that contain one proviral DNA per cell, kindly provided by NIH AIDS Research and Reference Program, NIAID, Rockville, MD, were used to quantitate the relative amount of HIV-gag DNA. As a control for PCR of genomic DNA, a 1000-bp fragment of human β-actin DNA was amplified using β-actin primer pair (Clontech, Palo Alto, CA).

2.6. Detection of RT activity

The RT and DNA polymerases examined in the present study were obtained as previously described (Ono et al., 1989). HIV-1 RT was purified from *Escherichia coli* harboring an expression plasmid for the precise coding sequence of the enzyme. The purified enzyme was a generous gift from Dr S.H. Wilson, NIH, MD. DNA polymerases α, β and γ were purified from KBIII cells, as previously described for DNA polymerase α (Matsukage et al., 1976), β (Ono et al., 1979), and γ (Yamaguchi et al., 1980) with some modifications. The reaction mixture for HIV-1 RT contained the following components: 50 mM Tris–HCl, pH 8.0; 3 μg/ml (rA)n·(dT)12–18 (base ratio, 2:1);10 μM [^{3}H]dTTP (400 cpm/pmol); 5 mM dithiothreitol (DTT); 50 mM KCl; 15% (v/v) glycerol; 5 mM $MgCl_2$. The reaction mixture for DNA polymerase α included the following: 50 mM Tris–HCl, pH 7.5; 80 μg/ml activated calf thymus DNA; 10 μM each of dATP, dCTP, dGTP and [^{3}H]dTTP (1000 cpm/pmol); 5 mM DTT; 15% (v/v) glycerol; 4 mM $MgCl_2$. The reaction mixture for DNA polymerase β contained the following: 50 mM Tris–HCl, pH 8.5; 30 μg/ml (rA)n·(dT)12–18 (base ratio, 1:2); 10 μM [^{3}H]dTTP (400 cpm/pmol); 5 mM DTT; 100 mM KCl; 15% (v/v) glycerol; 0.2 mM $MnCl_2$. The reaction mixture for DNA polymerase γ included the following: 50 mM Tris–HCl, pH 7.5; 11 μg/ml (rA)n·(dT)12–18 (base ratio, 10:1); 1 μM [^{3}H]dTTP (6000 cpm/pmol); 5 mM DTT; 70 mM KCl; 15% (v/v) glycerol; 0.1 mM $MnCl_2$. The reaction (50 μl total volume) was started by adding 5 μl of enzyme, the reaction mixture was incubated at 37°C for 30 min, and the reaction was stopped by adding 20 μl of 0.2 M EDTA and immersing the mixture in ice. Then a 50 μl aliquot of the mixture was transferred to a DE81 filter paper disc and processed for radioactivity counting as previously described (Lindell et al., 1970).

2.7. Flowcytometric analysis of CD4 antigen

A total of 1 000 000 CEM clone 5 cells were incubated with 10 μg/ml of baicalin for 1 h at 37°C. After the cells were washed with PBS (0.3%)/FCS (0.05%) azide solution twice, 2 μg of OKT4A monoclonal antibody (Coulter, Miami, FL) or normal mouse IgG were added to 1×10^6 cells, then incubated for 30 min on ice. After washing with PBS (0.3%)/FCS (0.05%) azide twice, FITC-goat anti-mouse IgG was added to a final dilution of 1:50 and the mixture was incu-

bated for 30 min on ice. The cells were washed twice and analyzed by flow cytometry on a Cytoace (Japan Spectroscopic, Tokyo, Japan).

2.8. Analysis of effect of baicalin on gp120-CD4 binding by ELISA

The effect of baicalin on gp120-CD4 binding was analyzed by an ELISA method. In brief, the following three experiments were carried out using a CD4 capture ELISA kit (Intracel, Cambridge, MA).

Experiment 1, baicalin and sCD4 were added simultaneously into a well of a gp120-coated microtiter plate.

Experiment 2, baicalin and sCD4 were preincubated together for 1 h and added together into a well of a gp120-coated microtiter plate.

Experiment 3, baicalin alone was added first into a well of a gp120-coated microtiter plate. After a 1-h incubation, the added baicalin was washed out from the well and then a mixture of baicalin and sCD4 was added into the same well.

ELISA was conducted essentially according to the instructions of the ELISA kit manufacturer except that baicalin was included in the assay system. Baicalin concentrations employed were 0.1, 0.3, 1.0, 3.0, 10, 30 and 100 μg/ml, i.e. the concentration range at which baicalin showed an anti-HIV activity in our PBMC assay system.

3. Results

3.1. Effect of baicalin on fresh PBMC infected with HIV-1

When PBMC were preincubated with baicalin at concentrations of 0.01–100 μg/ml, a concentration-dependent inhibition of HIV-1 replication was observed (Fig. 2a). On average, a 72% reduction of HIV-1 p24 antigen was detected after cells were pretreated with baicalin at 1.0 μg/ml for 1 h in multiple experiments and the 50% inhibitory concentration (IC_{50}) of baicalin was 0.5 μg/ml. At concentrations above 100 μg/ml, baicalin significantly affected cell viability (Fig. 2a). The suppressive effect of AZT was approximately 90% at concentrations above 0.1 μg/ml. In order to investigate whether baicalin has or has not a direct anti-HIV-1 effect on the virus, the following test was carried out. The virus was preincubated with baicalin for 1 h and infected with PHA-activated PBMC. The cell culture and assay procedure for anti-HIV activity were the same as already described. There was no inhibitory effect of baicalin on HIV-1 infection (Fig. 2b).

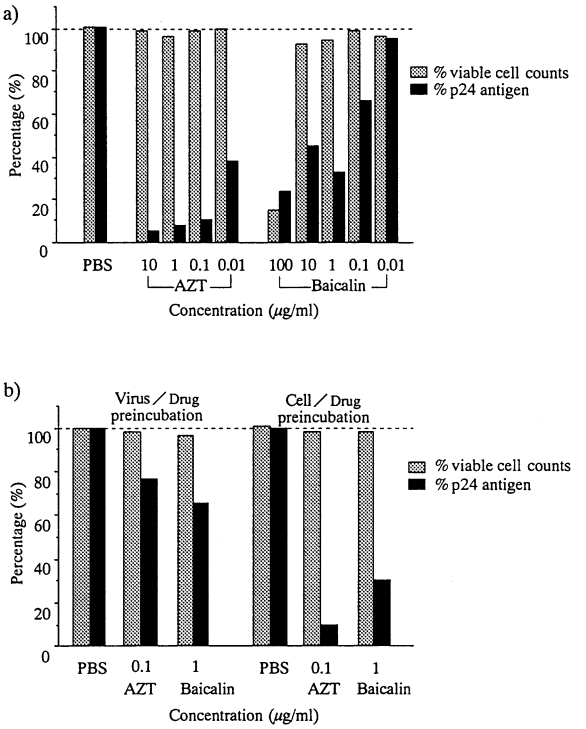

Fig. 2. Effect of baicalin on HIV-1 p24 antigen production in normal PBMC stimulated with PHA. (a) PHA-activated PBMC were preincubated in the presence of various concentrations of baicalin for 1 h and then infected with HIV-1. (b) HIV-1 was preincubated with baicalin for 1 h and then infected with PHA-activated PBMC.

3.2. Effect of baicalin on PBMC from HIV-1-infected individuals

At a concentration of 0.2 μg/ml, baicalin inhibited the production of HIV-1 p24 antigen by approximately 50%, and at 2 μg/ml inhibition by 90% (Fig. 3a). The minimum cytotoxic concentra-

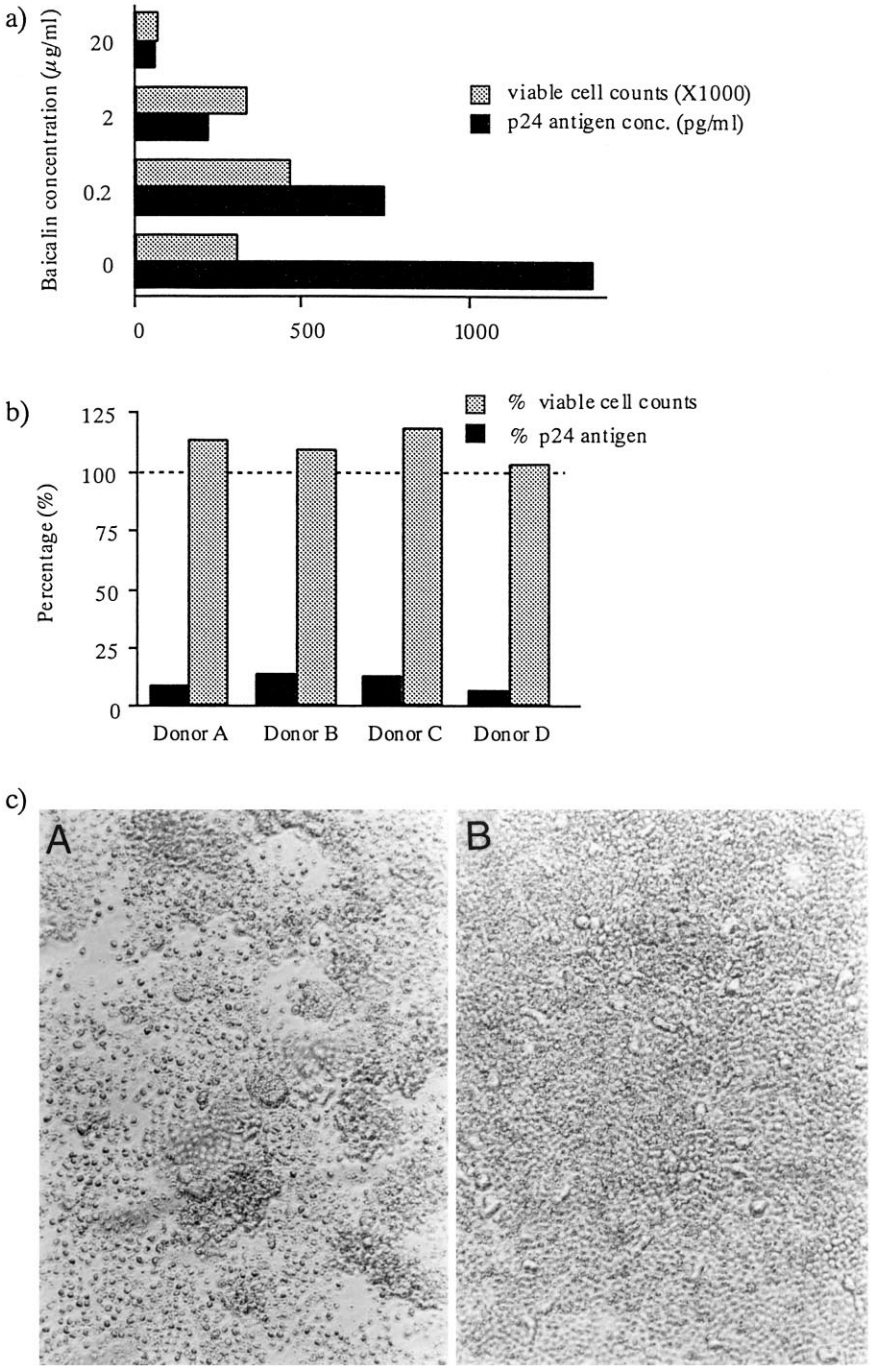

Fig. 3. Effect of baicalin on HIV-1 p24 antigen production in PBMC from asymptomatic HIV-1-seropositive individuals. (a) Various concentrations of baicalin were added to PBMC in the presence of PHA, and incubated for 7 days. HIV-1 p24 antigen concentrations of the culture supernatant and viable cell counts were determined by HIV-1 p24 antigen ELISA, and Trypan blue dye exclusion test, respectively. (b) Inhibitory effect of baicalin on HIV-1 p24 antigen production by using PBMC from other HIV-1-seropositive individuals. PBMC were cultured with 2 μg/ml baicalin or without baicalin in the presence of PHA for 7 days. HIV-1 p24 antigen concentration in the supernatant was quantitated using an ELISA assay. 100% values of HIV-1 p24 antigen concentration and viable cell count represent those without baicalin treatment. (c) Phase-contrast micrographs of PHA-activated PBMC from one of the HIV-1 infected individuals cultured for 7 days in the absence (A) or presence (B) of baicalin (2 μg/ml).

tion of baicalin was 20 μg/ml. AZT completely inhibited HIV-1 p24 antigen production at the concentration of 0.1 μg/ml (data not shown). Similar results were obtained with PBMC from four other HIV-1 carriers (Fig. 3b). Stimulation of PBMC with PHA led to cytopathic destruction of the cells (syncytia) after 7 days culture, as observed under microscopy (Fig. 3c-A). By adding 2 μg/ml baicalin to the culture medium, the cytopathic effect on the PBMC clearly decreased and the total cell number increased (Fig. 3c-B). The decrease of HIV-1 p24 antigen concentration in the culture supernatant was consistent with the decrease in syncytium counts. To examine the effect of baicalin on the viral DNA level, the genomic DNA of the PBMC was amplified by using HIV-specific primer pairs, and quantitated by a standard curve made with DNA from 8E.5 cells (Spear et al., 1990), known to contain one proviral copy per cell (Fig. 4A). Southern blot analysis of the PHA-activated PBMC from one of the asymptomatic HIV-1 carriers showed 500–1000 HIV-1-DNA copies per sample (Fig. 4B, lane 2). In contrast, the cells pretreated with baicalin (2 μg/ml) contained only 10–100 HIV-1 DNA copies per sample (Fig. 4B, lane 4). AZT-treated cells were also found to have a smaller number of HIV-1 DNA copies (average one to ten; Fig. 4B, lane 5). Normal PBMC (Fig. 4B, lane 3), and buffer alone (Fig. 4B, lane 1) were negative for HIV-1 DNA. All HIV-1 DNA samples from the PBMC examined for the effect of baicalin yielded similar PCR results (1000-bp band) with β-actin primer pairs (data not shown).

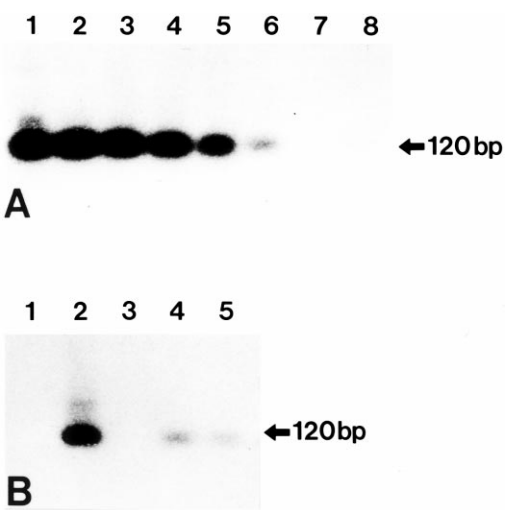

Fig. 4. Effect of baicalin on HIV-DNA copy numbers in PBMC activated with PHA and cultured for 7 days. (A) Concentration-dependent detection of amplified HIV-DNAs in the PHA-activated PBMC. 8E.5 cell numbers were adjusted and HIV-DNA was detected by PCR. Lanes 1-8 indicate 106, 105, 104, 103, 102, 101, 100 and 0 copies of virus DNA, respectively. (B) PCR products from genomic DNAs. Lane 1, buffer only; lane 2, PHA-activated PBMC of HIV-1 infected individual cultured for 7 days; lane 3, PBMC; lane 4, PHA-activated PBMC from the HIV-1 infected individual cultured for 7 days in the presence of 2 μg/ml baicalin; lane 5, PHA-activated PBMC of HIV-1-infected individuals cultured for 7 days in the presence of 0.1 μg/ml of AZT.

3.3. Detection of RT activity

To investigate the mechanism of the inhibitory effect of baicalin on HIV-1 production and HIV-1 transmission, we tested the effect of baicalin on HIV-1-induced cell fusion (Tochikura et al., 1988). In contrast to sulfated polysaccharides (Nakashima et al., 1987; Mitsuya et al., 1988; Baba et al., 1990), baicalin did not block syncytium formation of MOLT-4 cells with HTLV IIIB infected MOLT-4 cells at concentrations of 0.02–10 μg/ml (data not shown), suggesting that baicalin does not interfere with the binding of the viral envelope glycoproteins with the virus receptor on the cell surface. We therefore investigated the effect of baicalin on HIV-1 RT in our in vitro system (Ono and Nakane, 1990; Ono et al., 1990). At a concentration of 2 μg/ml, baicalin inhibited HIV-1 RT activity by approximately 50% and at 10 μg/ml baicalin inhibited the enzyme activity almost completely (Fig. 5). Inhibition of cellular DNA polymerases is an index for cytotoxicity. Although DNA polymerase β was slightly inhibited by baicalin, the DNA polymerases α and γ were not inhibited by the compound, indicating that the inhibitory effect of baicalin is specific for HIV-1 RT.

3.4. Binding ability of baicalin to CD4

The binding ability of baicalin to the gp120 binding site of the CD4 receptor on the surface of

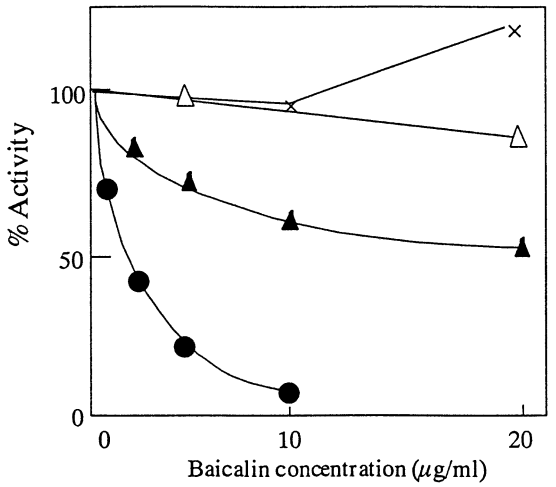

Fig. 5. Effects of baicalin on the activities of HIV-1 RT and DNA polymerases α, β and γ. The activities of HIV-1 RT (●) and DNA polymerases α (△), β (▲) and γ (X) were measured in the presence of various concentrations of baicalin. The 100% values (pmol) were 16.0 (●), 32.2 (△), 17.1 (▲), and 0.35 (X). All data correspond to a single assay for each point.

CEM clone 5 cells was also studied. The CEM cells clearly demonstrated specific surface fluorescence with the OKT4A mAb and the intensity was not changed by the pretreatment with 10 μg/ml of baicalin (data not shown).

3.5. Effect of baicalin on gp120-CD4 binding

To further investigate the inhibitory effect of baicalin on HIV adsorption, its effect on gp120-CD4 binding was examined using an ELISA system as described in Section 2. Three experiments were designed to distinguish the two possible mechanisms of interference with gp120-CD4 binding by baicalin, i.e. binding to CD4 and binding to gp120 (Section 2). Within the concentration range where baicalin showed a concentration-dependent anti-HIV-1 activity in our assay system using PBMC (Fig. 2a), no inhibitory effect of baicalin on gp120-CD4 binding was detectable in all three experiments conducted (Fig. 6). Based on these observations and the result of the binding assay of baicalin to CD4 presented in the preceding section, we conclude that the anti-HIV-1 activity of baicalin cannot be attributed to inhibition of virus adsorption to its target cell.

3.6. Toxicity and pharmacokinetics studies on baicalin

The toxicity and pharmacokinetics studies on baicalin are to be published elsewhere. Briefly, the oral acute toxicity (LD_{50}) in rats was higher than 2000 mg/kg. In the oral subacute toxicity study for 2 weeks, no abnormal change was noted even at a dose of 2000 mg/kg per day. Pharmacokinetic studies in rats carried out using labeled and non-labeled baicalin showed that C_{max} at 5 h after administration of baicalin (20 mg/kg) was 874.8 ng/ml plasma and the half-life was 11 h. The absolute bioavailability of baicalin calculated from the area under the time–concentration curve (AUC) was 62% (Wakui et al., 1992). In dogs, however, C_{max} at 1 h after oral administration of baicalin (100 mg/kg) was only 75.0 ng/ml, showing a considerable difference in its absorption and metabolism with respect to different animal species.

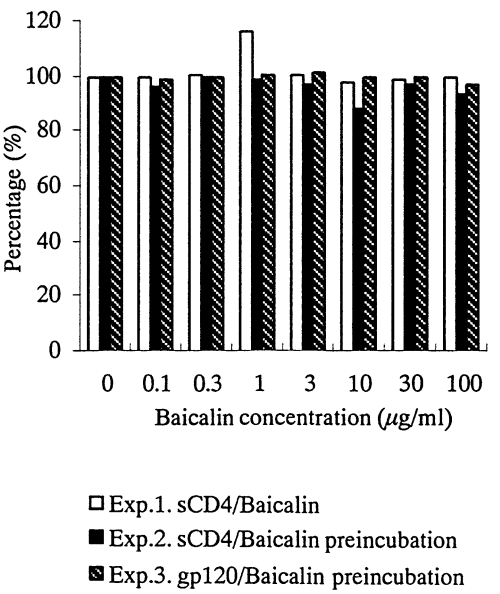

Fig. 6. Effects of baicalin on gp120-CD4 binding. Three experiments were carried out using CD4 capture ELISA kit.

4. Discussion

Baicalin was found to be a potent inhibitor of HIV-1 replication in fresh, PHA-activated, HIV-1-infected PBMC from healthy donors. The ability of baicalin to inhibit HIV-1 was also evaluated in PHA-activated PBMC from HIV-1-infected individuals. The average number of copies of HIV-1-DNA in purified lymphocytes and monocytes is approximately 100–140 copies/150 000 cells from asymptomatic HIV-1-seropositive individuals as determined by the PCR method (Spear et al., 1990). The decreased number of HIV-1 copies detected by PCR in both baicalin- and AZT-treated cultures indicates that the proportion of HIV-infected cells during the 7 days culture was significantly decreased. Alternatively, HIV-1-infected PBMC might have been destroyed by the exposure to baicalin or AZT in the presence of PHA for 7 days. An oriental remedy, Sho-saiko-to (SST) has been reported to suppress the production of HIV in mononuclear cells from asymptomatic HIV-seropositive individuals (Buimovici-Klein et al., 1990). The most active component of the remedy has been known to be baicalin and the estimated concentration of baicalin in SST was, by calculation, similar to the effective dose of baicalin in our experiments. The PBMC assay system proved useful not only in determining the production of HIV-1 by the patients' lymphocytes and monocytes but also in the screening of new anti-HIV agents.

As for the mechanism of the anti-HIV-1 effect of baicalin, we found that baicalin inhibits HIV-1 RT activity. Plant flavonoids, including baicalein, the aglucuronidic form of baicalin, have an inhibitory effect on various cellular DNA and RNA polymerases (Ono and Nakane, 1990). In the case of baicalein, the aglycon of baicalin, the mode of inhibition was of the competitive type (murine leukemia virus RT) (HIV-1 RT) with respect to the template·primer ((rA)n·(dT)12–18) or mixed type, suggesting that baicalin also inhibits HIV-1 RT activity by interfering with the binding of viral RNA to the RT molecule near the active site of the enzyme (Ono et al., 1989, 1990). Baicalin does not inhibit the activity of HIV-2 RT or murine leukemia virus RT (data not shown). Furthermore, baicalin neither inhibited the binding of OKT4A mAb to the gp120 binding site of CD4 nor interfered with the gp120-CD4 binding. This definitely rules out the possibility that baicalin interferes with the virus adsorption step.

Baicalin has been reported to inhibit HIV-1 LTR-directed reporter gene expression (Watanabe et al., 1994). This could be achieved by suppression of the signal transduction pathway induced by NFκB or a transcription regulatory factor. The detailed suppressive mechanism of the NFκB pathway by antioxidants has not been elucidated, but there is evidence that tumor necrosis factor may be involved in the regulation of the redox conditions of cysteine residues in NFκB (Hayashi et al., 1993). Binding of baicalin to the active site of HIV-1 RT may not be the only mechanism involved in the inhibition of HIV-1 replication in PHA-activated PBMC by baicalin. Further studies are necessary to clarify other possible mechanisms, such as an antioxidant activity, in the inhibitory effects of baicalin on HIV-1 production in PBMC.

To date, AZT, ddI, d4T and various other substances (De Clercq, 1995a,b) have been licensed for the treatment of HIV infection. Synergistic anti-HIV-1 effects of baicalin with AZT have been reported (Inada et al., 1994), suggesting that baicalin might be potentially useful as part of drug combination regimens for the treatment of HIV-1 infections.

Acknowledgements

We thank Dr Naoki Yamamoto, Department of Microbiology, Tokyo Medical and Dental University, Japan for helpful discussions. This work was supported in part by a Grant-in-Aid for Promotion of Human Science from the Japan Health Science Foundation.

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